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van den Bogaart, Geert; Krasnikov, Victor; Poolman, Bert

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Supplementary Information to

Dual-color fluorescence-burst analysis to probe protein efflux through the mechanosensitive channel MscL

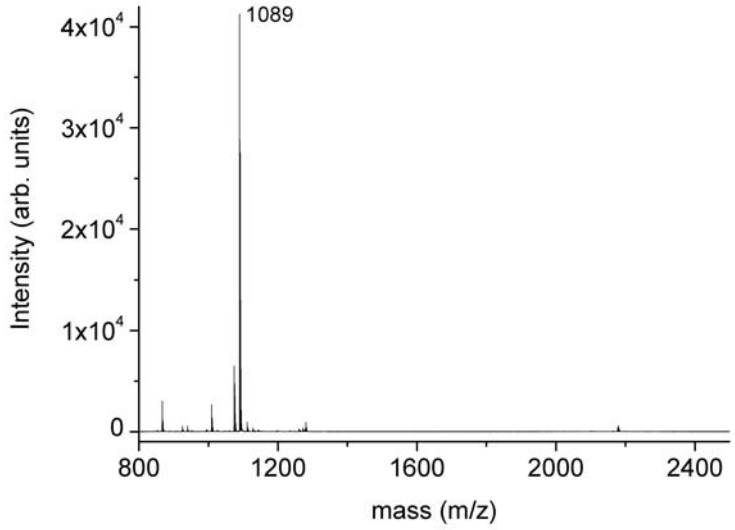
Geert van den Bogaart[*], Victor Krasnikov[†], and Bert Poolman[*‡]

*Biochemistry Department and †Ultrafast Laser and Spectroscopy Laboratory
Groningen Biomolecular Science and Biotechnology Institute & Materials Science Centre[plus]
University of Groningen
Nijenborgh 4, 9747 AG, Groningen, The Netherlands

‡to whom correspondence should be addressed:
phone: +31 50 363 4190
fax: +31 50 363 4165
E-mail: b.poolman@rug.nl

MASS OF ALEXA FLUOR 633

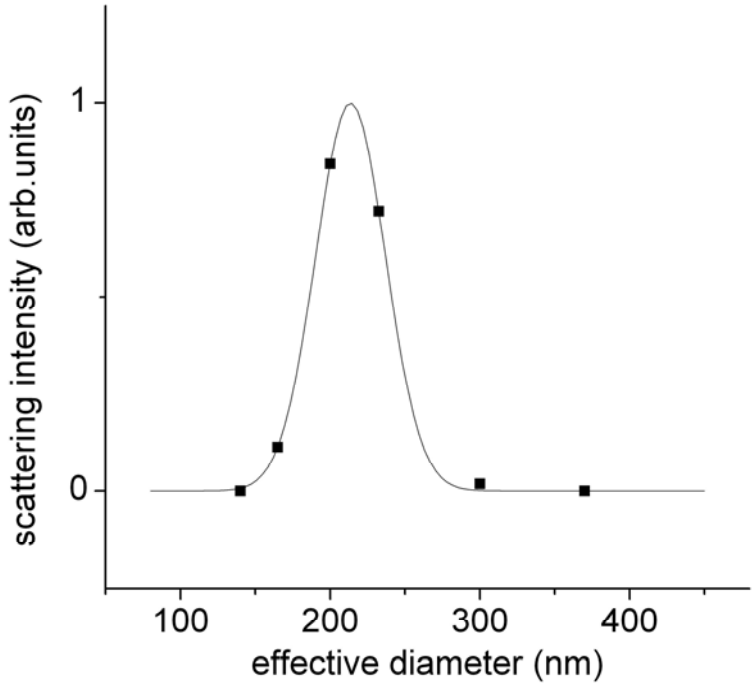
The mass of Alexa fluor 633 was determined using matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry. 0.5 µl of a 1 µM solution of Alexa fluor 633 in water with 0.1% TFA were spotted on the MALDI target and immediately mixed 1 : 1 with the matrix solution consisting of 10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (dissolved in 70% acetonitril plus 0.065% TFA). The spots were allowed to dry completely before the MALDI-TOF experiment was carried out on the Applied Biosystems 4700 Proteomics Analyzer. The mass of Alexa 633 was 1089 Da in both the unconjugated (supplementary figure 1) and the conjugated state (not shown).



Supplementary figure 1:
Mass of Alexa fluor 633 by MALDI-TOF.

SIZE OF THE LIPOSOMES

Dynamic light scattering (DLS) experiments were performed to determine the size of the liposomes. Supplementary figure 2 shows the result of DLS measurements on liposomes that were extruded through 200 nm diameter filters. An average radius of the liposomes of 107 ± 5 nm was found, with a spread of 16.5 ± 1.5 nm. The solid line is a fit, assuming a Gaussian distribution. DLS was performed at a fixed angle of 90° with a Malvern Instruments Sizer 5000, using the Contin analysis mode.



Supplementary figure 2:
Size of the liposomes by DLS.

FLUORESCENCE CORRELATION SPECTROSCOPY

To verify the results obtained by dual-color fluorescence-burst analysis, the diffusion of macromolecules was assessed using FCS. With FCS, one can determine diffusion constants and concentrations (1) for ensemble averages. The fluorescence autocorrelation signals of the DiO and the Alexa fluor 633 channels were measured and the data were fitted with a two-step analytical model to quantify the release of macromolecules. The fitting model for a normalized autocorrelation function of P different compounds, assuming 3-dimensional Brownian motion, is given by (2):

$$G_\tau = \frac{1}{V} \frac{\sum_{i \in P} C_i \alpha_i^2 \frac{1}{1 + \frac{4\tau D_i}{\omega_{xy}^2}} \frac{1}{\sqrt{1 + \frac{4\tau D_i}{z_0^2}}}}{\left(\sum_{i \in P} C_i \alpha_i \right)^2} \tag{1}$$

where α_i is the product of the fluorescence quantum yield and the absorption cross-section, C_i is the concentration of the molecules, ω_{xy} and z_0 are the effective radii perpendicular and parallel to the focal axis, respectively, and V is the effective confocal volume, defined as $V = \pi^{3/2} \omega_{xy}^2 z_0$.

Because not all liposomes were of the same size (Supplementary Fig. 2) and the diffusion constant is inversely related to their radius (Einstein-Stokes relationship), the fluorescence autocorrelation curves could not be fitted with equation 1 and assuming two components (Eq. 1, $P = 2$). Therefore, the model was modified so that it included the distribution of the liposomes. The radii of the liposomes follow a Maxwell distribution, which can be approximated with a Gaussian distribution, with average radius R and spread a. The fraction of liposomes with radius r is then given by:

$$k(r) = \frac{2 \exp\left(-(r-R)^2 a^{-2}\right)}{a\sqrt{\pi}\left(1 + \operatorname{erf}\left(\frac{R}{a}\right)\right)} \tag{2}$$

The number $W(r)$ of DiO molecules per liposome is related to the radius r by:

$$W(r) = \frac{8\pi c}{A} r^2 \tag{3}$$

where c is the ratio of DiO to lipid molecules and A is the surface area of a lipid (0.6 nm^2). The number of macromolecules S inside a liposome is related to the radius of the liposome by:

$$S(r) = \frac{4}{3} \pi C_S r^3 \tag{4}$$

where C_S is the concentration of substrate inside the liposome. The diffusion coefficient D of a liposome also depends on its radius according to the Einstein-Stokes relationship:

$$D(r) = \frac{\gamma}{r} \tag{5}$$

where γ is a constant determined by the viscosity and temperature of the medium.

Assuming that α_i is linearly dependent on the number of DiO or substrate molecules in the liposome, the fluorescence autocorrelation of DiO (Eq. *6*) and substrate (Eq. *7*) are given by:

$$G(\tau) = \frac{1}{VC_l} \frac{\int_0^\infty W(r)^2 k(r) \frac{1}{1+\frac{4\tau D(r)}{\omega_{xy}^2}} \frac{1}{\sqrt{1+\frac{4\tau D(r)}{z_0^2}}} \mathrm{d}r}{\left(\int_0^\infty W(r)k(r)\mathrm{d}r\right)^2} \tag{6}$$

$$G(\tau) = \frac{1}{V_r} \frac{fC_l \int_0^\infty S(r)^2 k(r) \frac{1}{1+\frac{4\tau D(r)}{\omega_{xy}^2}} \frac{1}{\sqrt{1+\frac{4\tau D(r)}{z_0^2}}} \mathrm{d}r + C_f \frac{1}{1+\frac{4D_f\tau}{\omega_{xy}^2}} \frac{1}{\sqrt{1+\frac{4D_f\tau}{z_0^2}}}}{\left(fC_l \int_0^\infty S(r)k(r)\mathrm{d}r + C_f\right)^2} \tag{7}$$

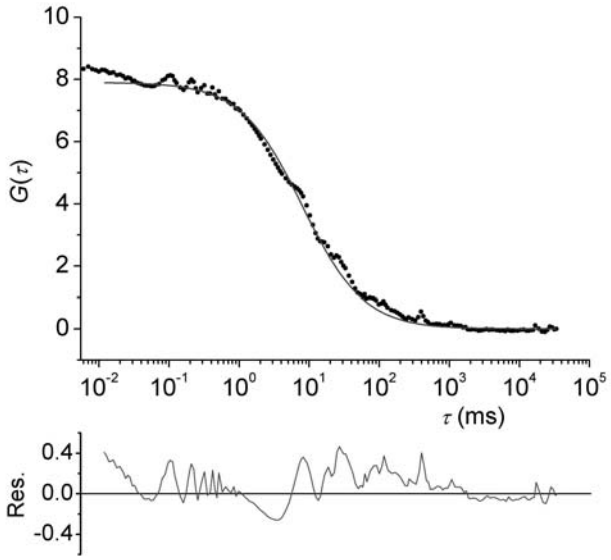
where C_l is the concentration of liposomes, D_f is the diffusion constant of the free substrate, C_f is the concentration of free label, and f is the fraction of liposomes containing substrate. If substrate effluxes through MscL, the percentage of fast component F increases after opening of the channel, where F is defined as:

$$F = \frac{C_f}{fC_l \int_0^\infty S(r)^2 k(r)\mathrm{d}r + C_f} \times 100 \tag{8}$$

The numerators of equations 6 and 7 cannot be analytically obtained and were therefore approximated numerically, using Simpson's method in 8 steps from $r = R - 2a$ to $r = R + 2a$.

To determine the spread in the sizes of the liposomes, fluorescence autocorrelation spectroscopy of liposomes containing the fluorescent lipid analogue DiO was performed. Fitting the autocorrelation function with equation 6 showed reasonable agreement (Supplementary Fig. 3). For coefficient γ (Eq. 5), a value of $2.3 \cdot 10^{-19} \pm 0.5 \cdot 10^{-19}$ m^3 s^{-1} was found, corresponding to a diffusion constant of $D \approx 2.3$ μm^2 s^{-1} for a liposome with a radius of 100 nm in 50 mM potassium phosphate, pH 7.0, plus 1 M glycerol. This D is about 60 times smaller than that of free Alexa fluor 633 which has a diffusion constant $D = 135 \pm 6$ μm^2 s^{-1} in 50 mM potassium phosphate, pH 7.0, plus 1 M glycerol. These diffusion constants are in accordance with the Stokes-Einstein

relationship. Furthermore, fitting the autocorrelation curves with equation 6 (Supplementary Fig. 3), yielded an average radius R of 112 ± 7 nm and spread a of 17.5 ± 2.5 nm. Both numbers are in excellent agreement with DLS measurements, where R = 107 ± 5 nm and a = 16.5 ± 1.5 nm were determined (Supplementary Fig. 2). For the measurements on Alexa fluor 633, The autocorrelation curves were fitted with Eq. 7 and the fits were acceptable when γ was given the same value as estimated from the measurements with DiO (Fig. 6).



Supplementary figure 3:
Autocorrelation curve of liposomes containing fluorescent lipid analogue DiO. The solid line represents a fit, using equation 6, with average radius R = 110 nm and spread a = 19 nm.

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